IOWA STATE UNIVERSITY
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Volume 31 | Issue 1 Article 4

1969

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Contrary to some conclusions of the lay public, rabies still constitutes a major problem to human and animal welfare. In fact in the last four or five years there has been somewhat of an increase in the incidence of rabies. The veterinarian serves a most important role in the control of rabies. He must necessarily be familiar with the disease, vaccination procedures which are employed, and the state daignostic laboratory facilities which are at his disposal. To be of greater service to his client the veterinarian should be aware of the manner in which a specimen should be submitted to the lab and the procedures that laboratories use in the examination of the submitted sample.

Submission of Specimens for Rabies Examination

The first and a most important factor influencing the accuracy of the rabies diagnosis is the manner in which the specimens are submitted. Specimens should be packaged and transferred to the laboratory in such a manner to assure a minimum amount of decomposition and time. Live suspects should be captured and held for observation by a veterinarian. If capture is not possible, the suspected animal should be killed in such a manner so as not to damage the brain. The head should be removed and placed in an airtight plastic bag; a second sealed plastic bag over the first is advisable as insurance against leakage. This should be refrigerated until ready for delivery but not frozen. (Freezing tends to break down the normal architecture of the brain.) Brain tissue in glycerine is unsatisfactory for fluorescent antibody (FA) examination.[3] For shipment the plastic bags containing the head should be packed into a container with crushed ice or other type of cold packaging material. During the summer at least 5 parts ice are usually required for 1 part of head. This package should be boldly labeled "Rabies Suspect" to warn those handling the parcel. During packaging and necropsy procedures, protective devices such as gloves and possibly eye glasses should, by all means, be used. A complete case history should always be included with the sample. This ought to include:

1. Date submitted
2. Dates of exposure and clinical observation
3. Species of the suspect
4. Names and addresses of:
   - Animal's owner or on whose land the exposure occurred
   - Person or persons exposed
     - Parents of children exposed
   - Physician
   - Veterinarian
   - Other persons suspected of being exposed
5. Description of clinical signs and the behavior of the rabies suspect

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6. Dates of first noticed signs and death of the animal including whether killed or natural death
7. Location, number, and severity of bites and scratches
8. Vaccination record: of specimen submitted, of animals exposed

Delivery of specimens to the lab by the people directly involved with the animal is usually the most desirable method. Samples may be brought at any time as refrigeration is provided outside the entrance for samples which arrive after lab hours. Samples may be sent by railway express but this has been found to be undesirable. Due to the long duration of time in shipment, most specimens arrive in conditions unsatisfactory for examination. Rabies specimens are not allowed to be sent through the U.S. Mail. The Iowa Highway Patrol will relay the specimens to the labratory only in an emergency involving human exposure.

The Iowa Veterinary Medical Diagnostic Laboratory at Ames and the Iowa State Hygienic Laboratory at Iowa City have prepared the following DON'T LIST which will provide guidance to anyone submitting a suspect specimen for examination:

1. Don't shoot or club the suspect specimen in the head. The animal's brain must be intact for proper laboratory examination.
2. Don't send the whole carcass to the laboratory. (Whole animals may be sent to the Iowa Veterinary Diagnostics Lab if examination for other diseases is desired.) The intact head is all that is needed for rabies examination. The State Hygienic Lab in particular does not have suitable facilities for the disposal of larger animal carcasses.
3. Don't fail to pack the head in ice immediately. Brain tissue rapidly decomposes if not properly refrigerated, particularly in summer months.
4. Don't freeze the specimen. Freezing alters brain tissue and makes it difficult to provide a proper diagnosis.
5. Don't fail to include with the specimen information concerning the exposure, name of person bitten, and the name of the physician or veterinarian to whom the laboratory report is to be submitted.
6. Don't use the Iowa Highway Patrol as a delivery service unless it is an absolute and constituted emergency. The only constituted emergency is when a person has been bitten by an animal likely to have rabies. Since mice and other rodents rarely, if ever, are infected with rabies, persons bitten by these animals do not constitute emergencies. Biting means the penetration of the skin by the teeth of the suspected animal. Bites about the head and neck, multiple and/or extensive bites elsewhere constitute an emergency. Salivary exposure without a bite is not an emergency.
7. Don't submit live animals for exami-

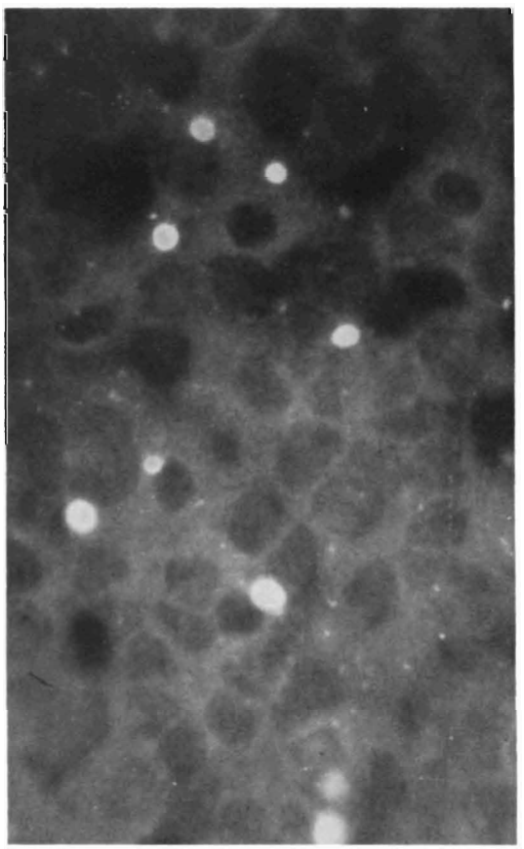

Figure 1. Impression smear of rabid brain tissue at a magnification of 540X with an ultraviolet light microscope. Note inclusion bodies with halo appearance. Courtesy of Dr. L. N. Brown.

nation. The laboratories do not have facilities for this service. Consult with your physician or veterinarian who will advise you on proper confinement procedures. If the animal is alive and appears healthy ten to fourteen days after the biting, it is proof that it did not have rabies in the infectious stage at the time of biting.

8. Don't kill the animal—unless confinement is impossible. Observation of the biting animal during confinement is superior in most instances to the application of the most advanced laboratory techniques.
9. Don't send specimens to the laboratory by mail. The U. S. Post Office refuses to handle rabies specimens. Bus or air shipment may be used. Preferably persons involved should deliver the head directly to the laboratory and make sure that sufficient ice is used to provide refrigeration during shipment.
10. Don't send specimens to the laboratory C.O.D. They will not be accepted.
11. Don't fail to include with the specimen, when available, a complete history of the animal including vaccinations administered, clinical signs, duration of illness and any postmortem findings.

Laboratory Procedure for Rabies Examination

The specimen and case history are recorded and necropsy performed. Due to greater virus concentrations a section of the hippocampus is removed from the brain; however, the cerebellum is used from bovine specimens. Brain tissue is formalinized for further histopathologic examination where indicated. Two impresion smears are made on each slide, one for the test, the other to serve as a control. Also two slides are prepared for each case, the second is stored 4 to 5 months for later reference. The slides are placed in acetone at —20° C in preparation for FA examination. The remaining portion of the hippocampus (approximately 1–1½ cm^3) is macerated in sterile water to be used for mouse inoculation (MI) and is also stored for approximately 5 months. To this suspension 10,000 units of penicillin and 10 mg of streptomycin are added to control bacteria.

The results of the FA test are recorded and sent by mail to the referring veterinarian and physician; if the specimen is positive and it involves human exposure, the result is telephoned immediately.

Mice are inoculated with the brain suspension for all FA negative cases which involve human exposure and for others in which verification of the FA test is desired. For each case six mice are inoculated intra-cranially on the midline just posterior to the ears with a minute amount (approximately 0.04 ml) of the brain suspension supernatant. These are observed daily for signs of illness and those which die 7 days post-injection are examined by FA test. (Less than 7 days is considered to be too brief for the normal viral incubation period and death is attributed to another cause.) Reports on the status of the mice are sent at 15 and 30 days post-injection to the referring veterinarian and physician. MI is not performed on cases diagnosed positive by the FA test.

Fluorescent Antibody Technique

Classically, rabies is diagnosed by the presence of Negri inclusion bodies in the neuronal cytoplasm, particularly those cells of the hippocampus and cerebellum, and by animal inoculation. Various differential stains of tissue smears have been used to demonstrate Negri body presence, such as Lenz, Sellers, or Giemsa and the staining of paraffin sections with hemotoxylin and eosin. However, since 1963 fluorescent antibody examination has become an accepted technique due to its rapidity and proven accuracy.

The staining conjugate of the FA test is prepared by "tagging" fluorescein isothyocyanate (a dye which fluoresces when exposed to ultra-violet light) to the rabies antibodies in the antiserum globulin. This is diluted with a suspension of normal mouse brain tissue to assimilate the control conjugate. Rhodamine is added to act in counterstaining the tissue of the smear;

TABLE I

Summary of Rabies Examinations at the State Hygienic Laboratory, 1938 to 1965, and Iowa Veterinary Medical Diagnostic Laboratory, 1951 to 1965[2]

Species	Total Examined	Negative	Positive No.	Positive %
Badger	30	28	2	6.7
Bat	430	417	13	3.0
Beaver	12	12	0	0.0
Bird	12	12	0	0.0
Cat	5850	5404	446	7.6
Chinchilla	4	4	0	0.0
Chipmunk	92	92	0	0.0
Civet cat	83	31	52	62.7
Cow	2892	1802	1090	37.7
Coyote	2	1	1	50.0
Deer	11	10	1	9.1
Dog	5139	4441	698	13.6
Ferret	7	7	0	0.0
Flying Squirrel	2	2	0	0.0
Fox	349	277	72	20.6
Goat	7	5	2	28.6
Gopher	196	194	2	1.0
Ground hog (woodchuck)	148	146	2	1.4
Ground squirrel	263	263	0	0.0
Guinea pig	77	77	0	0.0
Hamster	626	626	0	0.0
Hawk	4	4	0	0.0
Horse	163	96	67	41.1
Man	2	2	0	0.0
Mink	85	84	1	1.2
Mole	109	108	1	0.9
Monkey	24	23	1	4.2
Mouse	804	804	0	0.0
Mule	2	2	0	0.0
Muskrat	398	397	1	0.3
Opossum	183	183	0	0.0
Owl	12	12	0	0.0
Pig	246	187	59	24.0
Pocket gopher	3	3	0	0.0
Porcupine	1	1	0	0.0
Rabbit	696	694	2	0.3
Raccoon	913	880	33	3.6
Rat	689	687	2	0.3
Rodent	1	1	0	0.0
Sheep	96	87	9	9.4
Shrew	7	7	0	0.0
Skunk	2998	895	2103	70.1
Snake	1	1	0	0.0
Squirrel	2362	2342	20	0.8
Unknown	4	3	1	25.0
Vole	3	3	0	0.0
Weasel	21	21	0	0.0
Wolf	1	1	0	0.0
Total	26,060	21,379	4,681	18.0

TABLE II

Summary of Rabies Examinations at the Iowa Veterinary Medical Diagnostic Laboratory, 1966 through September, 1968

Species	Total Examined	Negative	No.	%
Bovine	439	364	75	17.1
Canine	314	304	10	3.2
Feline	1184	1130	54	4.6
Skunk	322	148	174	54.0
All others	2280	2251	29	1.3
Total	4539	4197	342	7.5

antibiotics are also added to the suspension to control contamination. This preparation is known as "a" conjugate or normal mouse brain (NMB).

A "b" conjugate of rabid mouse brain (RMB) is prepared in like manner except that the suspension to be used as the dilution factor is made from the brains of rabid mice. The virus antigens from the rabid mouse brain tissue combines with the antibodies of the rabies antiserum, thus preventing the staining of the inclusion bodies of the positive tissue smear. This conjugate serves in the FA test as a "control."

Two impression smears are made on each slide and the slides are fixed in acetone for a minimum of ten minutes, after which they are removed from the acetone and dried. One of the smears on the slide is stained with "a" conjugate (NMB); the other smear is stained with "b" conjugate (RMB). These are incubated in a humid atmosphere at 37° C for thirty minutes. The slides are then removed and washed in 0.01 M phosphate buffered saline for ten minutes to remove the excess conjugate. Following, the slides are rinsed in fresh distilled water, blotted dry, and cover slips are mounted.

Slides are studied at a magnification of 540X on a dark-field, ultra-violet microscope. The smear stained with "a" conjugate is first examined. The fluorescein labeled rabies antibodies should stain any rabies antigen that is present in the smear. The stained inclusion bodies will usually appear as bright green, round bodies of varying size. As may be noted in the photograph (Fig. 1), the bodies appear to have a halo or are more intense in their outer ring and less intense toward the center. On most positive samples the inclusion bodies are spread rather uniformly about the smear, and are quite readily discernible from other fluorescing non-specific material. Some positive specimens, however, may appear as little more than fine, dust-like, "sandy" green material; these may offer some difficulty to the examiner. The smear stained with "b" conjugate is examined next. Since this solution is made with rabid brain tissue, the rabies antigen should absorb the fluorescein-labeled antibody preventing any of the antibodies from staining the antigen material in the tissues. This portion of the test is useful in determining which of the fluorescing particles seen in the "a" smear are just nonspecific fluorescing material since antigen staining is blocked in the "b" smear. A positive interpretation is made when stained bodies of the characteristic morphology are seen in the smear stained with "a" conjugate, but are not seen in the "b" conjugate smear. A known positive control slide is routinely included with a group of slides as a check on the system.

In 1961 and 1962 the State Hygienic Lab conducted a study to evaluate the accuracy of methods of rabies diagnosis, particularly fluorescent antibody technique. Out of 457 total specimens 40 were diagnosed positive by FA test and 43 were positive on MI. The FA test was in agreement with 99.34% of the cases positive by MI.

Records of the Iowa Veterinary Medical Diagnostic Lab demonstrate that for the 1,983 cases submitted in 1967, 121 cases were diagnosed positive by FA technique and 122 by MI for an accuracy of 99.95%.

Summaries of the cases received at the two state laboratories are given in Tables I and II. From these one can get some idea of those species most commonly tested and the percent positive. For example, one might note the apparent effect that vaccination programs have had on canine rabies, and the very low incidence in rodents. Also, note the opossum, in which the disease has never been known to occur.[1]

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. M. W. Vorhies and Dr. L. N. Brown for their assistance in the preparation of this manuscript.

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